EXPERIMENTAL CELL RESEARCH 214, 486–498 (1994)

The Receptor for Urokinase-Type Plasminogen Activator of a Human Keratinocyte Line (HaCaT)

JEANNETTE REINARTZ,*,[1] JUTTA LINK,* ROBERT F. TODD,† AND MICHAEL D. KRAMER*

*Institut für Immunologie und Serologie der Universität Heidelberg, Laboratorium für Immunpathologie, Im Neuenheimer Feld 305, 69120 Heidelberg, Germany; and †University of Michigan Medical Center, Division of Hematology/Oncology, 3119 Taubman Center, 1500 East Medical Center Drive, Ann Arbor, Michigan 48109-0374

It is assumed that plasmin participates in pericellular proteolysis in the epidermis. Plasmin is generated by keratinocyte-associated plasminogen activators from the proenzyme plasminogen; plasminogen activation can proceed at the keratinocyte surface. The resultant plasmin interferes with cell to matrix adhesion and does possibly contribute to keratinocyte migration during reepithelialization. Here we describe the receptor for urokinase-type plasminogen activator (uPA-R) in the human keratinocyte cell line HaCaT, which serves to direct plasminogen activation to the cell surface; we relate the receptor to the uPA-R previously described in human myelo-/monocytes. Binding of uPA to the receptor accelerated plasminogen activation by a factor of ≈10, compared to uPA in solution. Receptor-bound uPA was susceptible to inhibition by the plasminogen activator inhibitors 1 and 2. uPA and uPA-R antigen, as well as uPA activity, were localized to the leading front of expanding sheets of HaCaT cells. Exposure of HaCaT cells to plasminogen was followed by detachment of the cells. Detachment was prevented by an anticatalytic anti-uPA antibody, by the plasmin-specific inhibitor aprotinin, and by the lysine analogue tranexamic acid, the latter of which prevents plasmin(ogen) binding to the cell surface. Our findings support the hypothesis that uPA-mediated plasminogen activation is characteristic of mobile rather than sessile keratinocytes. Moreover, the uPA-R seems to focalize plasminogen activation to the surface of cells at the site of keratinocyte migration.

INTRODUCTION

Plasminogen activation by epidermal plasminogen activators is thought to be involved in pericellular proteolysis under physiological and pathological conditions [1–3]. These conditions include epidermal differentiation [4], reepithelialization during wound healing [5], autoimmune blistering skin diseases [6–8], or psoriasis [9, 10]. Keratinocytes are the major cellular constituent of the epidermis. Recently, we have described in human keratinocytes a cell surface-associated pathway of plasminogen activation that can provide plasmin for pericellular proteolysis [11]. Activation of plasminogen depended on the binding of exogenous plasminogen to low-affinity binding sites at the keratinocyte surface and was mediated by surface-bound urokinase-type plasminogen activator (uPA)[2] [11]. To avoid variations caused by different strains of cultured keratinocytes we used the HaCaT cell line for our analysis, which has originally been established from keratinocytes of adult trunk skin [12]. The HaCaT cells were spontaneously immortalized, retained a high degree of epidermal differentiation [12], and duplicate normal human epidermal keratinocytes in many functions, including the capability for uPA-dependent plasminogen activation at the cell surface [11].

From previous studies of others [13, 14] it was concluded that uPA-mediated plasminogen activation may interfere with cellular adhesion to the extracellular matrix and may thus favor the mobilization of keratinocytes under conditions of wound healing [5], and possibly also under conditions of epidermal blister formation [14]. A cell surface receptor for uPA (uPA-R) expressed by migrating rather than by sessile keratinocytes appeared to play a crucial role in directing uPA activity to the cell surface [15, 16]. From all previous studies it was

[1] To whom correspondence should be addressed. Fax: ++49/6221/564030.

[2] Abbreviations used: DSS, disuccinimidyl suberate; dNTP, nucleotide triphosphate; FCS, fetal calf serum; HMW-uPA, high-molecular-weight uPA; LMW-uPA, low-molecular-weight uPA; mAb, monoclonal antibody; PA, plasminogen activator; PAI(-1 or -2), plasminogen activator inhibitor (-1 or -2); PCR, polymerase chain reaction; PE, phycoerythrin; PI-PLC, phosphoinositol-specific phospholipase C; plasmin(ogen), this term is used when the respective method, e.g., detection by antibodies, cannot discriminate between plasminogen and plasmin; pro-uPA, proenzyme of uPA; (pro-)uPA, this term is used, when the respective method, e.g., detection by antibodies, cannot discriminate among pro-uPA, HMW-uPA, and/or LMW-uPA; RT, room temperature; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPA-R, uPA receptor (CD 87).

0014-4827/94 $6.00

of interest to explore in detail the enzymatic consequences and cell-biological implications of the uPA/uPA-R interaction in human keratinocytes.

Accordingly, the studies presented herein were performed (i) to identify the uPA binding protein(s) (uPA-R) of the keratinocyte cell line HaCaT, (ii) to explore the molecular requirements for the interaction between the receptor and the ligand, (iii) to determine the functional implications of uPA binding to its receptor, (iv) to study the regulation of receptor-bound uPA by plasminogen activator inhibitors, (v) to disclose the topography of uPA-R expression and uPA-mediated plasminogen activation in HaCaT cell cultures, and finally (vi) to link uPA-mediated plasminogen activation with plasmin-mediated mobilization of HaCaT cells in culture.

MATERIALS AND METHODS

Materials. The plasmin-specific chromogenic substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251; No. 41206) and plasminogen (20 U/mg; No. 41304) were from Haemochrom Diagnostika (Essen, FRG). High-molecular-weight (54 kDa) uPA (Ukidan) was obtained from Serono (Freiburg, FRG), LMW-uPA (33 kDa) from Sigma (Deisenhofen, FRG), PAI-1 from American Diagnostica (via Ortho Diagnostic systems, Neckargemünd, FRG), and recombinant PAI-2 from the Behringwerke Ag (Marburg, FRG). Human recombinant pro-uPA was kindly provided by Grünenthal (Aachen, FRG). The monoclonal antibodies against uPA (HD-UK 1), the uPA-R binding domain of uPA (anti-ATF Peptide, HD-UK 2) [17], and the uPA-R (3B10) [18] have recently been described. Polyclonal goat anti-uPA, anti-tPA, anti-PAI-1, anti-PAI-2, and rabbit anti-uPA-R [18] were purified from whole serum by affinity chromatography (Protein G). Biotin-labeled goat anti-rabbit IgG, biotin-labeled rabbit anti-goat IgG, and Cy3-labeled streptavidin were obtained from Dianova (Hamburg, FRG).

All other reagents for molecular biological techniques were purchased from Boehringer (Mannheim, FRG). Ultrapure dNTP-Mix was from Pharmacia (Freiburg, FRG), RNA isolation kit was provided by Stratagene (Heidelberg, FRG). Phosphoinositol-specific bacterial phospholipase C was purchased from Boehringer.

Buffer salts and detergents of analytical grade were from Merck (Darmstadt, FRG). DME medium and all medium supplements were from Seromed (Berlin, FRG). The serum-free HL-1 medium was from Paesel (Frankfurt/M., FRG).

Cell culture and removal of noncovalently surface-associated molecules. The human keratinocyte cell line HaCaT [12] was cultured at 37°C in a humidified atmosphere of 7% CO_2 using DME medium supplemented with 2 m*M* L-glutamin and 10% (v/v) heat-inactivated fetal calf serum. For serum-free cultures the cells were propagated in HL-1 medium. In control experiments the HL-1 medium was found to be suited for keratinocyte culture while not influencing the immunological and functional determination of plasminogen activators.

Molecules associated with the cell surface in a noncovalent manner were eluted by incubation with glycine buffer (50 m*M* glycine/HCl, pH 3.0, 100 m*M* NaCl) for 3 min at RT. Subsequently, the cells were neutralized with Hepes buffer (0.5 *M* Hepes/NaOH, pH 7.5, 100 m*M* NaCl) and immediately resuspended in PBS.

Chromogenic substrate assay on cultured cells. HaCaT cells were seeded at a density of 5×10^4/well into flat-bottom 96-well tissue culture plates and were cultivated in serum-free HL-1 medium for 18 h. The cultures were washed twice with PBS and then taken for enzymatic analysis. PA activity was determined by addition of plasminogen (0.05 U/ml) and the chromogenic plasmin substrate S-2251 (40 μg/ml) dissolved in HL-1 medium. The cells were incubated at 37°C, and after different time intervals the absorbance change at 405 nm was directly determined from the 96-well plate by using an ELISA reader. The assay was performed in the presence of various additions: different concentrations of tranexamic acid (0.6–15 m*M*), PAI-1 (0.2–50 μg/ml), or PAI-2 (0.01–10 μg/ml). In certain experimental setups a constant amount of PAI-1 or PAI-2 (5 μg/ml), respectively, was preincubated with goat anti-PAI-1 IgG, goat anti-PAI-2 IgG, or normal goat IgG (1–250 μg/ml) before addition to the cells.

To evaluate the initial rate of plasminogen activation by cell-associated or soluble uPA, HaCaT cells were grown to confluence in microtiter tissue culture plates in DMEM containing 10% FCS [19]. The cells were acid-stripped and incubated with 3 n*M* uPA for 30 min at RT. The enzyme reaction was started by the addition of various concentrations of plasminogen (12–380 n*M*) and the chromogenic plasmin substrate S-2251 (0.3 m*M*). The plasmin hydrolysis of S-2251 was monitored continuously at 405 nm. As a control the experimental assay was performed in parallel without HaCaT cells.

Initial velocities were calculated from the slope (b) of the plots of the absorbance at 405 nm versus time^2 by using the equation $v_i = b(1 + K_m/S_o)/\epsilon k_e$ [19], where K_m is the apparent Michaelis constant of S-2251 hydrolysis by plasmin (0.3 m*M*), k_e is the empirically determined catalytic rate constant for plasmin hydrolysis of S-2251 (2.5×10^4 *M* min^{-1} *(mol of plasmin)* $^{-1}$), and ϵ *is the molar extinction coefficient* of *p*-nitroanilide at 405 nm (10^4 M^{-1} cm^{-1}) [20].

Ligand binding of the HaCaT cell uPA-R. HaCaT cells were seeded at a density of 5×10^4/well into flat-bottom 96-well tissue culture plates and were cultivated in serum-free HL-1 medium for 18 h. The cells were incubated for 3 min with glycine buffer followed by neutralization with Hepes buffer to remove endogenously bound uPA. Cells were washed twice with PBS and incubated with HMW-uPA (0.061–1 μg/ml) or LMW-uPA (1 μg/ml). Alternatively, the incubation with HMW-uPA was performed in the presence of the following mAb (100 μg/ml): anti-ATF (HD-UK 2; IgG_1), anti-uPA-R (3B10; IgG_2). The anti-Borrelia burgdorferi antibody LA 18 (IgG_1) and LA 1 (IgG_2) served as irrelevant isotype-identical control antibodies [21]. After washing twice in PBS, the cells were taken for analysis of PA activity by addition of plasminogen and the chromogenic plasmin substrate S-2251 as described above.

Polymerase chain reaction. Total cellular RNA was isolated from cells by the method of acid guanidinium thiocyanate/phenol/chloroform extraction [22]. Total cellular RNA (1 μg) was transcribed into cDNA in a total volume of 100 μl containing 200 μ*M* dNTP-Mix, 25 U RNase inhibitor 2 μ*M* dithiothreitol, 5 U AMV reverse transcriptase, 0.2 μ*M* of each 5′ (^{121}CATGCAGTGTAAGACCAACG^{141}) and 3′ (^{432}CTCTCACAGCTCATGTCTGATGAGCCACAGGAAATGC^{396}) primer in a buffer of 10 m*M* Tris, pH 8.3, 1 m*M* $MgCl_2$, 50 m*M* KCl 0.01% gelatine. The primer sequences were derived from the uPA-R cDNA sequence [23]. The mixture was overlaid with mineral oil. The reverse transcription was carried out at 42°C for 1 h, terminated by heating to 95°C for 5 min, and then quick-chilled on ice. The polymerase chain reaction was then started by addition of 2.5 U *Taq* polymerase. Thirty-five rounds of amplification were done in the DNA Thermal cycler (Biomed, FRG) with a 94°C denaturation step (30 s), 45°C annealing step (45 s), and 74°C extension step (90 s). After amplification, the product of each reaction was subjected to electrophoresis in a 1.5% agarose gel in TBE buffer. The bands were visualized by ethidium bromide staining. The identity of the PCR amplification products were verified by sequencing (data not shown).

Radioactive ligand binding. Human recombinant pro-uPA was labeled with ^{125}I by Immundiagnostik GmbH (Bensheim, FRG) to a specific activity of 4.9 μCi/μg by using the chloramine-T method.

To study radioactive ligand binding, HaCaT cells were grown to confluence in 24-well tissue culture plates in DMEM containing 10% FCS. Cells were washed three times with ice-cold 0.1% BSA/PBS, and afterward 0.2 ml 0.1% BSA/HL-1 containing increasing amounts

of [^{125}I]pro-uPA was added. The plates were incubated on ice for up to 120 min. After three washes with ice-cold PBS the monolayers were solubilized with 0.2 ml 0.1 *M* NaOH and radioactivity was counted in a gamma-counter. Specific binding was determined by measuring the difference in cell-bound radioactivity in the absence or the presence of excess unlabeled pro-uPA. The cell number was determined in parallel by cell counting after having detached the cells from the plate by trypsin treatment. Scatchard analysis was used to quantitate the number of binding sites and K_d of the complex [24].

Flow cytofluorometry. To circumvent the effect of proteolytic enzymes which can affect the uPA-R [25] single cells were obtained as follows: cultured HaCaT cells were detached by trypsinization and seeded in regular serum-containing culture medium. After 4 h incubation under normal culture conditions the keratinocytes could be detached as single cells by incubation with 0.2% EDTA/PBS. For detection of the uPA-R, cells were first acid-treated as described above in order to unmask uPA-R possibly saturated with endogenous ligand. Then, 2×10^5 cells were incubated with anti-uPA mAb (HD-UK 1; undiluted serum-free culture supernatant) or anti-uPA-R mAb (3B10; 1:100 dilution of ascites fluid) for 1 h on ice in a total volume of 100 μl 1% BSA/PBS. Alternatively, cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC; 1 U/ml, 30 min, 37°C) prior to the incubation with anti-uPA-R mAb. After extensive washing with 1% BSA/PBS the cells were incubated with 2 μg/ml of phycoerythrin (PE)-labeled species-specific goat anti-mouse IgG $(Fab')_2$ in 1% BSA/PBS (30 min, on ice). After extensive washing the cells were resuspended and fixed in PBS containing 1% formaldehyde. Labeled cells were analyzed by flow cytofluorometry (FACScan, Becton–Dickinson, Heidelberg).

To determine the autocrine saturation of the uPA-R, acid-treated cells were quickly neutralized and then incubated in HL-1 medium at 37°C. After different times of incubation, one-half of the cells were stained for uPA and the other half for the uPA-R.

Immunocytology. For immunostaining, HaCaT cells were cultured on glass coverslips. After 24 h, cultures were washed twice with PBS and fixed with ice-cold 70% ethanol. Then they were incubated for 60 min at RT in 10 μg/ml affinity-purified goat anti-uPA IgG (10 μg/ml) or rabbit anti-uPA-R IgG (20 μg/ml). In the second step, the bound antibodies were detected by biotin-labeled goat anti-rabbit or rabbit anti-goat IgG (1:250), respectively, followed by visualization of bound biotin with Cy3-labeled streptavidin (1:1000). The negative controls that were included in our immunostaining procedures were as follows: (i) omission of the first antibody by PBS and (ii) replacement of the first specific poly- or monoclonal antibody by either isotype-identical irrelevant monoclonal antibodies (anti-Borrelia burgdorferi antibodies [21]) or by nonimmune polyclonal IgG preparations. These controls did not yield positive staining results (data not shown). After mounting of stained sections in PBS/glycine, photomicrographs were taken using a Zeiss microscope with an integrated camera and an Ektachrom 400× (Kodak Ltd.) color slide film.

Overlay zymography. The functional activity of the plasminogen activators was studied by zymography on HaCaT cell cultures. The overlay mixture consisted of 0.5 ml 8% (w/v) low-fat dry milk powder solution, 0.75 ml PBS (0.9 m*M* Ca^{2+} and 1 m*M* Mg^{2+}), 0.7 ml 2.5% agar solution in water, and 20 μl of a 4-mg/ml solution of purified human plasminogen. Control experiments were carried out with overlay mixtures from which plasminogen was omitted. The overlay mixture was prepared at 50°C and 180 μl was mounted on the cell layer and spread evenly under glass coverslips. Zymograms were allowed to develop at 37°C for 24–48 h, and photographs were taken under dark-ground illumination. To distinguish uPA- from tPA-catalyzed plasminogen activation, anti-catalytic goat anti-uPA or anti-tPA antibodies (100 μg/ml) were added to the overlay mixture.

Cross-linking of uPA and uPA-R. HaCaT cells were grown to confluence on tissue culture plates and freed from endogenously bound uPA as described above. The cells were incubated with 1 n*M* [^{125}I]pro-uPA in the absence or the presence of excess unlabeled pro-uPA (2 h, on ice). After extensive washing, surface-bound proteins were covalently cross-linked by treatment with disuccinimidyl suberate (DSS; 1 m*M* final concentration; incubation for 15 min at RT) [26]. Control cells were not treated with DSS. The reaction was stopped by the addition of ammonium acetate (100 m*M* final concentration, 15 min at RT). Cells were lysed in 1 ml 1% (v/v) Triton X-100/PBS (30 min on ice) and electrophoretically separated by using a 10% SDS–polyacrylamide gel under nonreducing conditions followed by autoradiographic analysis.

Detachment of cultured cells. HaCaT cells were grown to confluence on tissue culture plates and treated without or with plasminogen (0.5 U/ml) in Ca^{2+}- and Mg^{2+}-free PBS. Alternatively, the plasminogen treatment was performed in the presence of various additions: tranexamic acid (15 m*M*), aprotinin (100 U/ml), anti-catalytic goat anti-uPA, or anti-tPA antibodies (100 μg/ml). After 4 h of incubation, the cells were fixed with 2.5% glutardialdehyde for 15 min at RT, followed by three washes with 0.1 *M* borate buffer (pH 8.5). Cells were then stained with methylene blue (1 mg/ml) in borate buffer (pH 8.5, 15 min at RT). Microphotographs were taken using a Kodak T-Max black and white film of 400 ASA.

RESULTS

Characterization of the uPA-R Expressed by HaCaT Cells

Previously, a uPA receptor of human myelo-/monocytes (uPA-R) has been identified, cloned, and sequenced [23, 25, 27]. To determine whether HaCaT cells and normal human epidermal keratinocytes express a similar uPA-R, the expression of uPA-R-specific mRNA was studied. Total cellular RNA of HaCaT cells and normal human epidermal keratinocytes was isolated, cDNA was synthesized by reverse transcription, and a PCR was performed by using oligonucleotides specific for the myelo-/monocytic uPA-R. RNA derived from the myelo-/monocytic cell line U 937 (Fig. 1A, lane 2), which are known to express the uPA-R [28, 29], served as positive control. RNA of the human NK cell line YT [30], which is negative for the uPA-R [our unpublished observations] (Fig. 1A, lane 5) served as negative control. A reaction product having the expected molecular weight of 312 bp indicated the expression of uPA-R-specific mRNA in U 937 cells, HaCaT cells (Fig. 1A, lane 3), normal human epidermal keratinocytes (Fig. 1A, lane 4), but not in YT cells. No specific product was obtained in any cell when the PCR was performed without reverse transcription; identity of the amplification product was proven by cloning and sequencing (data not shown).

To explore whether HaCaT cells express a functionally active uPA-R, ligand binding and cross-linking experiments with [^{125}I]pro-uPA were carried out. Cultured HaCaT cells were saturated with [^{125}I]pro-uPA and afterward treated with the cross-linking agent DSS. Surface-bound [^{125}I]pro-uPA was analyzed by SDS–PAGE and autoradiography. When the cells were saturated in the presence of an excess of unlabeled pro-uPA, no surface-associated [^{125}I]pro-uPA was found (Fig. 1B, lane

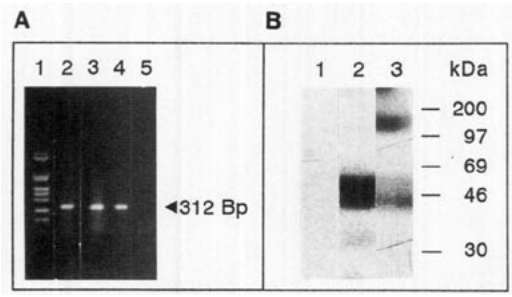

FIG. 1. Identification of uPA-R on HaCaT cells. (A) uPA-R-specific mRNA in HaCaT cells. Total cellular RNA (1 μg) was reverse transcribed into cDNA and amplified by using uPA-R-specific sense and antisense primers. The amplification product of 312 basepairs (bp) could be detected after separation by agarose gel electrophoresis (arrow). Lane 1, molecular weight marker; lane 2, U 937; lane 3, HaCaT cell line; lane 4, normal epidermal keratinocytes; lane 5, YT cell line. (B) chemical cross-linking of uPA-R and [^{125}I]pro-uPA. HaCaT cultures were freed from endogenously bound uPA by acid treatment. Afterward, the cells were incubated with a trace amount of [^{125}I]pro-uPA (1 n*M*) in the absence or the presence of excess unlabeled pro-uPA followed by DSS-mediated chemical cross-linking. The cells were solubilized and separated by SDS–PAGE under nonreducing conditions using a 10% gel. Radioactively labeled proteins were visualized by autoradiography. Lane 1, HaCaT cells incubated with [^{125}I]-pro-uPA in the presence of excess unlabeled pro-uPA; lane 2, HaCaT cells incubated with [^{125}I]pro-uPA without chemical cross-linking: only the 54-kDa band of free [^{125}I]pro-uPA was visible; lane 3, HaCaT cells incubated with [^{125}I]pro-uPA followed by chemical cross-linking: an additional band of 110 kDa is visible.

1). In cells that were saturated but not treated with the cross-linking agent DSS, the 54-kDa band of [^{125}I]pro-uPA was observed (Fig. 1B, lane 2). When, however, cells were saturated with [^{125}I]pro-uPA followed by treatment with DSS, an additional band of 110 kDa was observed (Fig. 1B, lane 3). The latter finding indicated covalent cross-linking of [^{125}I]pro-uPA (having a molecular weight of 54 kDa) with a binding structure of $\approx$55 kDa. The molecular weight corresponded to the known molecular weight of the uPA-R of myelo-/monocytes [27].

These findings indicated the presence of a uPA-R molecule in HaCaT cells that is identical to the uPA-R of myelo-/monocytes [28, 29]. Recently, a mAb specific for the uPA-R of activated macrophages has been developed (mAb "3B10") [18, 31]. To further identify the uPA-R molecule expressed by HaCaT cells, the mAb was used for flow cytometry (Fig. 2). The majority of the HaCaT cells stained positive with mAb 3B10 (Fig. 2A). When the cells were pretreated with the bacterial phosphoinositol-specific phospholipase C the staining was reduced in $\approx$50% of the cells to almost background levels (Fig. 2C). Partial susceptibility to PI-PLC has also been described for the uPA-R of myelo-/monocytes [31, 32].

To explore whether the uPA-R of HaCaT cells was saturated with (endogenous?) (pro-)uPA, the cells were analyzed by flow cytofluorometry using the (pro-)uPA-specific mAb HD-UK 1 [17]. The majority of the cell population displayed positive staining (Fig. 2B), indicating that the natural ligand (pro-)uPA was bound to the HaCaT cell surface.

Based on the hypothesis that the uPA-R is saturated by endogenous (pro-)uPA, the time course of uPA-R saturation with endogenous (pro-)uPA after removal of cell surface-associated (pro-)uPA was studied. The HaCaT cells were treated with acidic buffer to release (pro-)uPA from its receptor. Afterward, the cells were incubated for different time intervals in serum-free medium and then tested for surface-associated uPA and uPA-R by using the respective mAbs. The positive/negative cutoff was arbitrarily set to a fluorescence intensity of 5; cells were scored positive if they displayed a fluorescence intensity of >5. The fraction of uPA-R-positive cells was $\approx$80% during the entire observation period (open circles; Fig. 2D). The percentage of uPA-positive cells increased from $\approx$35% immediately after acid treatment of the cells to $\approx$65% after 190 min of incubation in serum-free medium (closed circles; Fig. 2D). The findings indicated a constant level of uPA-R expression and a time-dependent increase in uPA-R occupancy.

The physicochemical characteristics of the interaction between uPA and its receptor were explored next. The time dependence (Fig. 3A) as well as the concentration dependence (Fig. 3B) of [^{125}I]pro-uPA binding to HaCaT cells was studied. Radiolabeled pro-uPA bound to HaCaT cells and the interaction was inhibited by $\approx$75% by an excess of unlabeled pro-uPA, indicating that the binding was specific and saturable. The binding reached an equilibrium after $\approx$100 min at 4°C (Fig. 3A). When incubated with 15 n*M* [^{125}I]pro-uPA under conditions of apparent equilibrium binding (120 min, 4°C), HaCaT cells saturably bound $\approx 1.7 \times 10^4$ pro-uPA molecules per cell (Fig. 3B; Inset), which is comparable to other epidermal cell lines [33]. The binding interaction had an apparent K_d of 1 n*M*, which is in the similar range as for the uPA-R of myelo-/monocytes [34] and another epidermal cell line [33].

Structural Requirements for Binding to the uPA-R and Functional Activity of uPA-R-Bound uPA

HaCaT cells were grown to near confluence, acid-treated, and then saturated with graded concentrations of HMW-uPA (1–0.061 μg/ml) or a high concentration of LMW-uPA (1 μg/ml). After 1 h at RT the nonbound uPA was removed by washing. Plasminogen activation by the cell-bound uPA was then measured by addition of plasminogen and the plasmin-specific chromogenic peptide substrate S-2251. Without saturation, the cells activated the plasminogen to some extent, as indicated by an absorbance change of 0.2 after 60 min of incubation (Fig. 4A; open circles). Whether the activity was

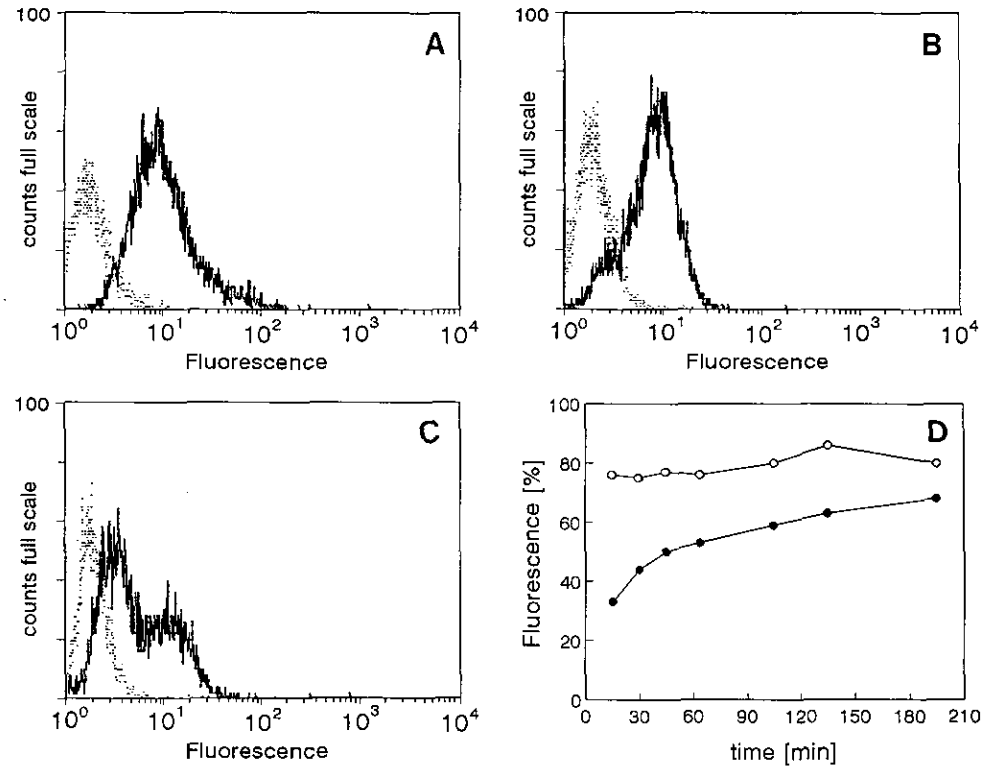

FIG. 2. The human keratinocyte cell line HaCaT expresses a uPA-R and cell surface-associated uPA as revealed by immunostaining with monoclonal antibodies and flow cytofluorometry. (A) Single cell suspensions of HaCaT cells were stained for uPA-R by using an anti-uPA-R-specific mAb (3B10) and (B) for cell surface-associated uPA by using an anti-uPA mAb (HD-UK 1). (C) HaCaT cells were incubated with PI-PLC (1 U/ml, 30 min, 37°C) prior to staining with the anti-uPA-R-specific mAb. Bound antibody was visualized by using phycoerythrin-labeled anti-mouse IgG. The dotted line depicts the negative control: HaCaT cells stained with phycoerythrin-labeled antibody only. (D) Autocrine saturation of uPA-R. HaCaT cells were freed from surface-bound uPA by acid treatment and incubated in serum-free HL-1 medium. After different times of incubation, the cells were stained for uPA-R (open circles) and uPA (closed circles), respectively. The data are shown as percentage of stained cells ("% positive cells"). The positive/negative cutoff was arbitrarily set to a fluorescence intensity of 5; cells were scored positive if they displayed a fluorescence intensity of >5.

due to incomplete removal of surface-associated uPA by the acid-treatment or to resaturation of the uPA-R with endogenous (pro-)uPA cannot be determined on the basis of our data. When, however, the cells were saturated with HMW-uPA, increased plasminogen activation was observed (Fig. 4A; closed symbols). The increase of plasminogen activation depended upon the concentration of HMW-uPA; 1 μg/ml of HMW-uPA yielded an absorbance change of 1.0 after 60 min of incubation. Preincubation of the cells with 1 μg/ml of LMW-uPA did not increase cellular plasminogen activation over background levels (compare open circles and rectangles in Fig. 4A). The findings indicated that the known receptor binding epitope in the growth factor domain of uPA, which is present in HMW-uPA but lacking in LMW-uPA, is involved in binding of (pro-)uPA to the HaCaT cell uPA-R.

Furthermore, we tested whether the binding interaction between HMW-uPA and the uPA-R could be blocked either by an anti-(pro-)uPA mAb that recognizes the receptor binding domain of (pro-)uPA (mAb HD-UK 2) [17] or by the anti-uPA-R mAb 3B10 that interferes with (pro-)uPA binding to the uPA-R [18, 35]. Binding of HMW-uPA to the HaCaT cells could be blocked by both antibodies, but not by isotype-matched control antibodies (Fig. 4B). The findings further corroborate that uPA is bound to the HaCaT cell uPA-R via the receptor-binding domain located within the growth factor domain of the A-chain of HMW-uPA and pro-uPA.

Efficiency of Plasminogen Activation by Receptor-Bound uPA

The data presented so far indicates that in HaCaT cells the uPA-dependent surface-associated plasminogen activation is mediated by uPA bound to a receptor molecule (uPA-R); the uPA-R is molecularly and functionally indistinguishable from the known uPA-R of myelo-/monocytes [27, 28, 36]. Next we explored whether the uPA-mediated plasminogen activation is altered when the enzyme is bound to the uPA-R. There-

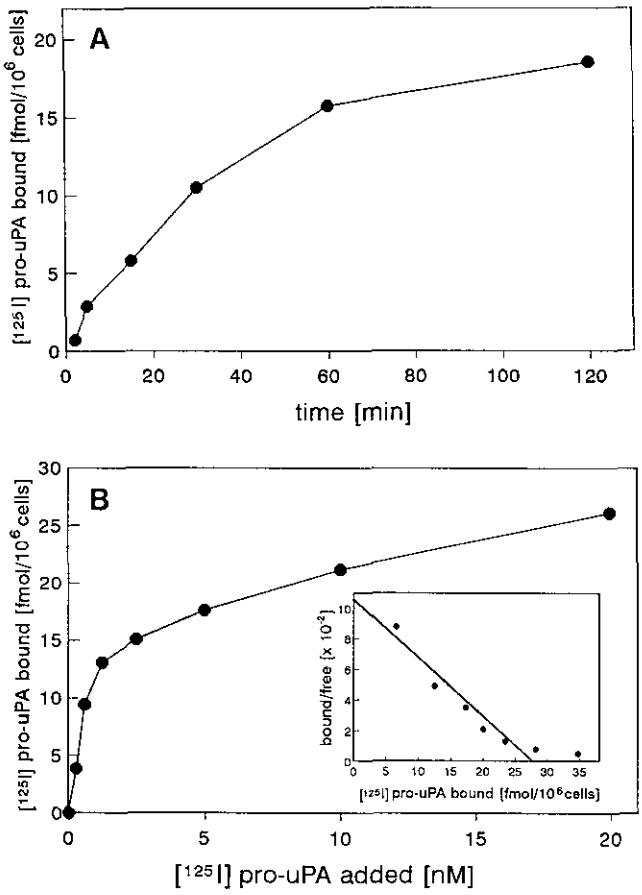

FIG. 3. Binding of [^{125}I]pro-uPA to HaCaT cells. (A) Time-dependent binding. HaCaT cells were incubated for different intervals of time with a trace concentration of [^{125}I]pro-uPA (1 n*M*) in the absence or the presence of an excess of unlabeled pro-uPA. For the indicated time points specific binding of [^{125}I]pro-uPA was calculated as follows: "specific binding" = (binding in the absence of unlabeled pro-uPA) − (binding in the presence of unlabeled pro-uPA). The specific binding was about 75% of the total binding. (B) Concentration-dependent binding. Acid-stripped cells were incubated at 4°C for 2 h with increasing concentrations of [^{125}I]pro-uPA (0.3–20 n*M*) in the absence or the presence of an excess of unlabeled pro-uPA. Specific binding was calculated as described above. Inset: Scatchard plot of pro-uPA binding to HaCaT cells. The data are derived from B. The best straight line fit to the experimental points was calculated by linear regression and had a coefficient of correlation of 0.96. The apparent number of [^{125}I]pro-uPA receptors per cell was 1.7×10^4 and the binding interaction had an apparent K_d of 1 n*M*.

fore, plasminogen activation in the presence of uPA-R-expressing HaCaT cells was studied essentially as previously described [37]. HaCaT cells were saturated with HMW-uPA and the activation of glu-plasminogen was followed by hydrolysis of the plasmin-specific substrate S-2251. The activation of plasminogen was enhanced by a factor of ≈7–10 in the presence of HaCaT cells (Figs. 5A and 5B). When plasminogen activation was studied in the presence of tranexamic acid, which prevents plasmin(ogen) binding to the cells, no enhancement was observed (Fig. 5A, closed circles). Tranexamic acid did not inhibit plasminogen activation by uPA in the absence of cells (Fig. 5A, closed rectangles).

Regulation of Receptor-Bound uPA

PAI-1 and PAI-2 are natural inhibitors of uPA activity [38, 39]. Both types of PA inhibitors are expressed in cultured human epidermal keratinocytes [14, 15], in HaCaT cells [Reinartz *et al.*, unpublished observations], and in the epidermis [40, 41]; the inhibitors may therefore act as regulators of keratinocyte-associated plasminogen activators. We explored whether uPA, when bound to the uPA-R of keratinocytes, is susceptible to regulation by the inhibitors. HaCaT cultures were incubated with graded concentrations of PAI-1 and PAI-2.

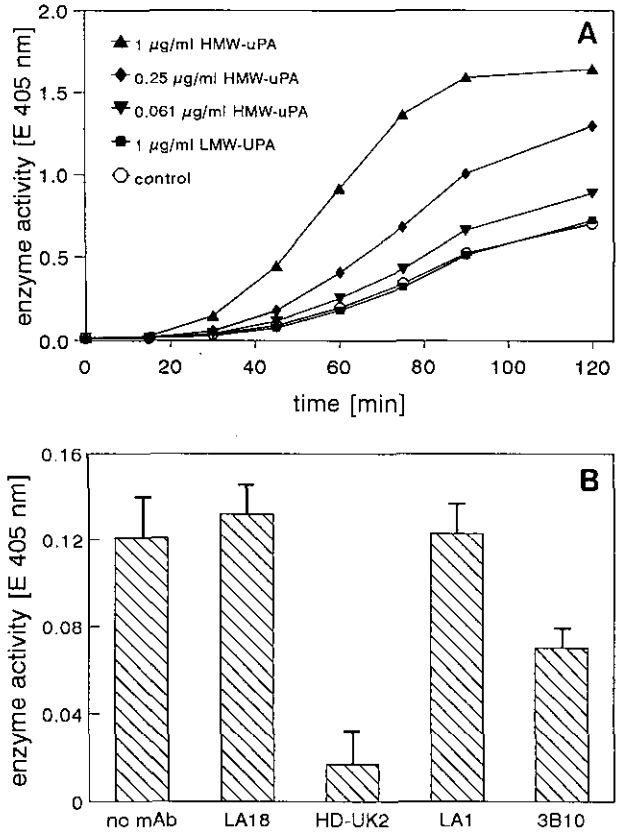

FIG. 4. Activity of cell surface-bound uPA. (A) Acid-treated HaCaT cells were incubated with HMW-uPA (0.061, 0.25, 1 μg/ml) or LMW-uPA (1 μg/ml) for 1 h. After extensive washing the cell-associated uPA activity was assessed in an indirect chromogenic assay using exogenous plasminogen and the plasmin-specific chromogenic peptide substrate S-2251. Substrate hydrolysis was followed at 405 nm for 120 min. (B) The saturation of acid-stripped cells with HMW-uPA (0.25 μg/ml) was performed in the presence of various monoclonal antibodies (200 μg/ml). The binding of HMW-uPA was reduced in the presence of a mAb recognizing the uPA-R-binding epitope of uPA (HD-UK 2) or an anti-uPA-R mAb (3B10), but not in the presence of isotype-matched control antibodies (LA18, LA1).

Plasminogen activation was then measured by addition of plasminogen and the plasmin substrate S-2251. PAI-1 (Fig. 6A) as well as PAI-2 (Fig. 6B) caused a dose-dependent inhibition of uPA-catalized plasminogen activation. The specificity of the interaction was proven by immunological blocking experiments, in which the inhibitory activity of the PAIs was counteracted by antibodies specific for PAI-1 or PAI-2, respectively (Figs. 7A and 7B).

Localization of uPA-Dependent Plasminogen Activation in HaCaT Cell Cultures

In view of the hypothesis that uPA [5, 42] and uPA-R expression [15, 33] is most pronounced in migrating keratinocytes, we tested the plasminogen activator activity in HaCaT cell cultures by overlay zymography (Fig. 8). Plasminogen-dependent lysis of the overlying protein gel was found to be most prominent at the leading edge of expanding HaCaT cell monolayers (Figs. 8B–8E). Moreover, lysis was most pronounced in rapidly expanding small clusters of HaCaT cells (Fig. 8D). Inhibition of plasminogen-dependent lysis by anti-uPA but not by anti-tPA antibodies, proved that lysis was uPA-dependent (Figs. 8E and 8F). In the next experiments we explored whether uPA activity colocalizes with uPA and uPA-R antigen. uPA (Figs. 9A–9C)- and uPA-R-specific (Fig. 9D) immunostaining was most pronounced at the leading edge of expanding keratinocyte sheets.

Taking the hypothesis that plasmin, which is generated in the pericellular space, can lead to the destabilization of cell/matrix interactions and favor the mobilization of keratinocytes [11, 16] we tested whether uPA-dependent generation of plasmin can cause the detachment of HaCaT cells from their culture substra-

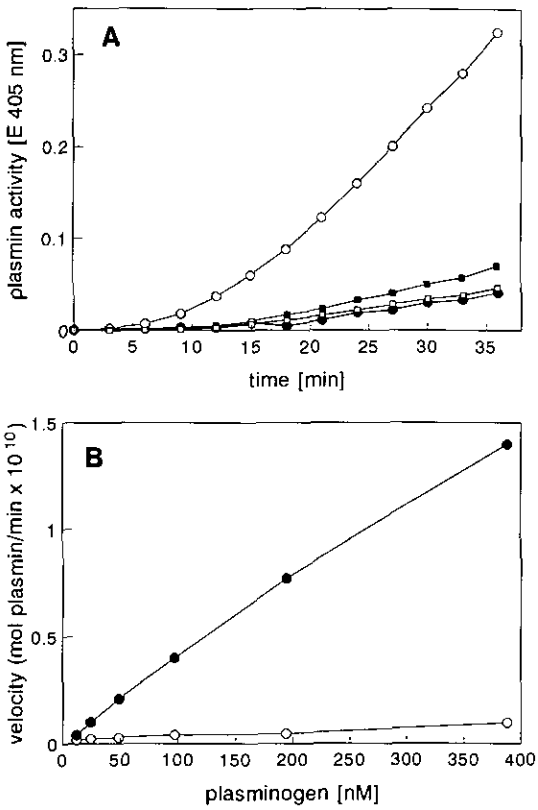

FIG. 5. Plasminogen activation by cell-associated or soluble uPA. HaCaT cells were grown in flat-bottom tissue culture plates. At subconfluence, cells were acid-stripped and incubated with 3 n*M* uPA for 30 min at RT. Afterward, varying concentrations of plasminogen (12–380 n*M*) and the plasmin-specific chromogenic substrate S-2251 were added. (A) Time-dependent plasminogen activation by uPA in the absence (open rectangles) or the presence (open circles) of HaCaT cells. The activation of plasminogen (380 n*M*) was measured at 3-min intervals and is given as absorbance change at 405 nm. Closed symbols represent parallel assays performed in the presence of 15 m*M* tranexamic acid. (B) Concentration- dependent plasminogen activation by uPA in the absence (open circles) or the presence (closed circles) of HaCaT cells. Initial velocities were determined from the slopes of plots of A_{405} versus time^2 as described under Materials and Methods.

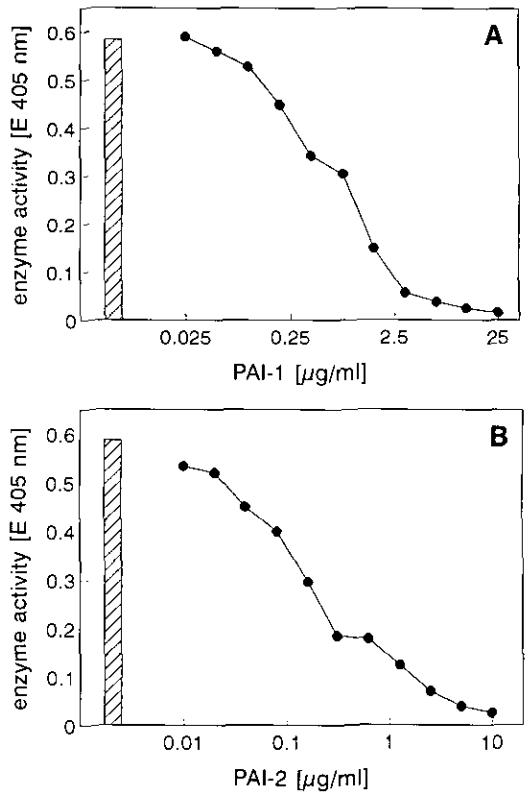

FIG. 6. Inhibition of cell-associated uPA by PAI-1 (A) and PAI-2 (B). HaCaT cells were grown in flat-bottom tissue culture plates. At subconfluence cells were incubated for 30 min at RT without (A, B, hatched bars) or with increasing concentrations of PAI-1 (A, closed circles) or PAI-2 (B, closed circles), respectively. PA activity was determined by addition of plasminogen and the plasmin-specific substrate S-2251 followed by incubation at 37°C. The hydrolysis of S-2251 was measured after 120 min at 405 nm.

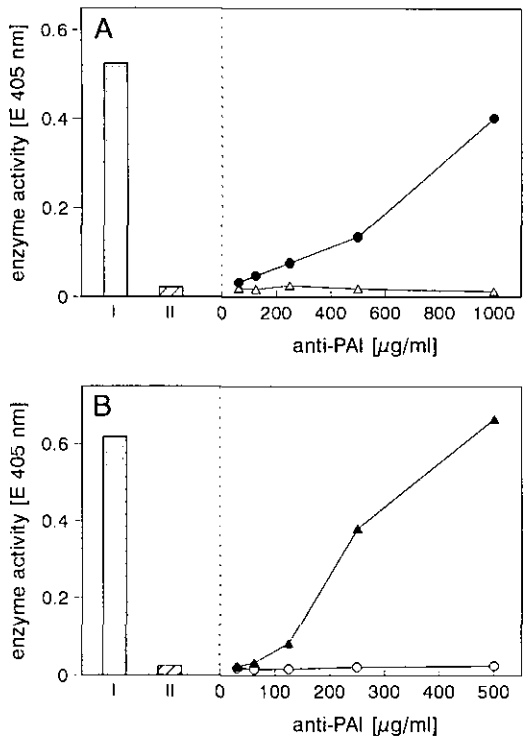

FIG. 7. The inhibitory activity of PAI-1 and PAI-2 is prevented by specific anti-PAI-1 or anti-PAI-2 antibodies, respectively. HaCaT cells were grown in flat-bottom tissue culture plates to subconfluence. Control (I): plasminogen activation by cells incubated without any additions; control (II): plasminogen activation by cells incubated with a standard concentration of 5 μg/ml PAI-1 (A) or PAI-2 (B). For the antibody inhibition experiments, PAI-1 (A) or PAI-2 (B) were incubated with increasing amounts of goat anti-PAI-1 IgG (triangles) or anti-PAI-2 IgG (circles) for 30 min at RT and then added to the cells. After 30 min at RT, PA activity was determined by addition of plasminogen and the plasmin-specific substrate S-2251. The hydrolysis of S-2251 at 37°C was measured after 120 min at 405 nm.

tum. HaCaT cells exposed to plasminogen under serum-free culture conditions for 0–4 h changed morphology to a more rounded shape and were partially detached from the culture plate (Fig. 10). Rounding and detachment was prevented if the incubation with plasminogen was performed in the presence of the plasmin inhibitor aprotinin (Fig. 10C) or in the presence of the lysine analogue tranexamic acid (Fig. 10D). In view of the previous findings that tranexamic acid prevents binding and subsequent activation of plasminogen at the keratinocyte surface [11], the present data indicate that the rounding and detachment of HaCaT cells depends on plasmin that is generated from plasminogen at the HaCaT cell surface. Moreover, detachment was prevented by anti-catalytic anti-uPA (Fig. 10E) but not by anti-catalytic anti-tPA (Fig. 10F) antibodies.

DISCUSSION

Previous studies had shown that plasmin is generated by uPA at the surface of normal human epidermal keratinocytes and the keratinocyte cell line HaCaT [11]. The expression of a uPA binding molecule(s) (uPA-receptor) that directs uPA activity to the cell surface appeared to be crucial for that process. Here we demonstrate that the keratinocyte cell line HaCaT expresses a high-affinity cell surface receptor for uPA (uPA-R) with a molecular weight of ≈55 kDa, a conclusion that is based on the Scatchard analyses (Fig. 3B), and the cross-linking experiments (Fig. 1B) using [^{125}I]pro-uPA. The molecular weight and affinity of the HaCaT cell uPA-R are similar to those of the uPA-R previously described in myelo-/monocytes [23, 28, 34, 36] and human keratinocytes [33, 43]. Identity with the myelo-/monocytic uPA-R was further suggested by the following evidence: (i) mAb 3B10, which has originally been raised against the uPA-R of activated macrophages [18, 31], reacted with HaCaT cells in flow cytofluorometry (Fig. 2) and inhibited uPA binding to the HaCaT cells (Fig. 4B), (ii) the PCR analyses using oligonucleotides specific for the myelo-/monocyte uPA-R [23] led to the expected amplification product with RNA from HaCaT cells and normal human epidermal keratinocytes (Fig. 1A), (iii) similar to the uPA-R of myelo-/monocytes [44] and normal keratinocytes [15], uPA binding to the uPA-R of HaCaT cells was of high affinity (Fig. 3B) and was mediated by the receptor-binding epitope located in the growth factor domain, which is present in HMW-uPA and pro-uPA, but lacking in LMW-uPA (Fig. 4A), and (iv) similar to the uPA-R of myelo-/monocytes, the HaCaT cell uPA-R was partially sensitive to PI-PLC (Fig. 2D). The collective evidence strongly suggests that HaCaT cells express a uPA-R that corresponds to the uPA-R of the myelo-/monocytes [27, 28, 36] and normal human keratinocytes [15].

In epidermal (patho)physiology, uPA-mediated plasminogen activation has been linked with the migration of keratinocytes during reepithelialization in wound healing [5]. Keratinocyte migration from the wound edge or from the hair follicles begins hours after wounding and precedes the enhanced cell proliferation required to restore epidermal architecture [15]. Plasminogen activation by uPA at the leading edge of migrating epidermal sheets may be advantageous to aid the keratinocyte in its movement through the complex extracellular matrix of the wound bed [45]. Several events in this process might be mediated by plasmin, e.g., cleavage of a path through the matrix, breakage of cell/substratum bonds to allow mobilization of keratinocytes and cell movement, and/or generation of biologically active fragments of fibrin or fibronectin [46, 47, 48]. In line with these considerations, McNeill and Jensen [15] explored primary cultures of neonatal foreskin keratinocytes: by radioactive ligand binding and by using anti-uPA antibodies for immunocytology they found that uPA-binding is enhanced at the edge of expanding keratinocyte sheets [15]. Our findings are in agreement with

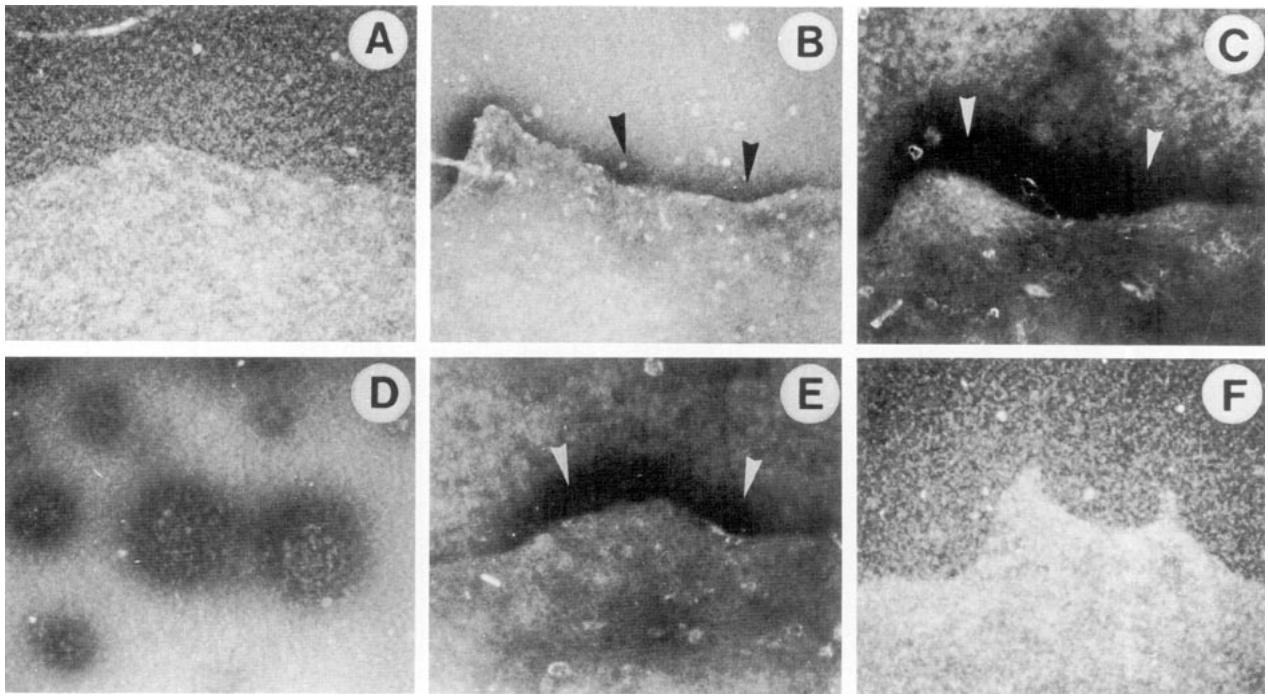

FIG. 8. Plasminogen activation by HaCaT cell cultures. Overlay zymography by using a plasminogen-containing indicator gel. Zymography was performed in the absence (A) or the presence (B, C, D, E, and F) of plasminogen. Furthermore, anti-catalytic goat anti-tPA (E) or goat anti-uPA (F) were incorporated into the indicator gel. After incubation for 48 h at 37°C plasminogen activation is reflected by dark clear zones of lysis (arrowheads). Microphotographs were taken using a Kodak T-Max black and white film of 400 ASA (magnification: ×100).

these previous data: in the HaCaT cells uPA-R as well as uPA antigen was up-regulated at the leading edge of expanding cell sheets (Fig. 9) and in small, rapidly expanding cultures (Fig. 9B), both locations where cell migration occurs. Thus, the data presented herein indicate that HaCaT cells duplicate normal keratinocytes in the regulation of uPA-R expression. The immunocytological findings on the localization of uPA were corroborated by zymography, showing that uPA activity was pronounced at—if not confined to—the leading edge of growing keratinocyte sheets and to small cell clusters (Fig. 8).

Our data (Fig. 2B) indicate that only part of the uPA-R can be removed by PI-PLC treatment. This is reminiscent of previous findings with myelo-/monocytes [31, 32] and does not necessarily indicate the existence of an additional subpopulation of transmembrane-anchored uPA-R molecules or an alternative binding mechanism for surface-associated uPA. The incomplete release of uPA-R observed after PI-PLC treatment may merely reflect a partial acylation of the 2-hydroxyl group of the inositol residue in the glycolipid anchor of the uPA-R, a modification found in some GPI-anchored proteins that hampers catalytic mechanism of PI-PLC [32]. It remains open, however, whether the fraction of the cell-bound uPA which is not releasable by PI-PLC treatment may be bound by alternative mechanisms, which could encompass binding to plasma membrane gangliosides [49].

With the observation that receptor-bound uPA is internalized only very slowly in neonatal foreskin keratinocytes, McNeill and Jensen [15] had already suggested that receptor-bound uPA serves an extracellular function. The assumption is supported and extended by our present findings. Plasminogen activation in the presence of uPA-R-expressing cells was increased by a factor of 7–10; no such stimulation was observed when the binding of plasminogen to the cell surface was prevented by tranexamic acid. This finding indicates that binding of uPA to the uPA-R not only serves to focalize uPA activity, but also to enhance its catalytic efficiency. The blocking of the uPA function by an anti-catalytic mAb, as well as the inhibition of plasmin by aprotinin, and blocking of plasminogen binding to the cell surface by tranexamic acid prevented the rounding and detachment of cultured HaCaT cells (Fig. 10) in the presence of plasminogen. These findings lend support to the hypothesis that receptor-bound uPA mediates activation of exogenous plasminogen and that the plasmin generated serves an extracellular proteolytic function that interferes with cellular adhesion to the growth substratum. The finding is compatible with previous data obtained by immunocytology on fibrosarcoma cells [50, 51] and electron microscopy on an epidermal cell line

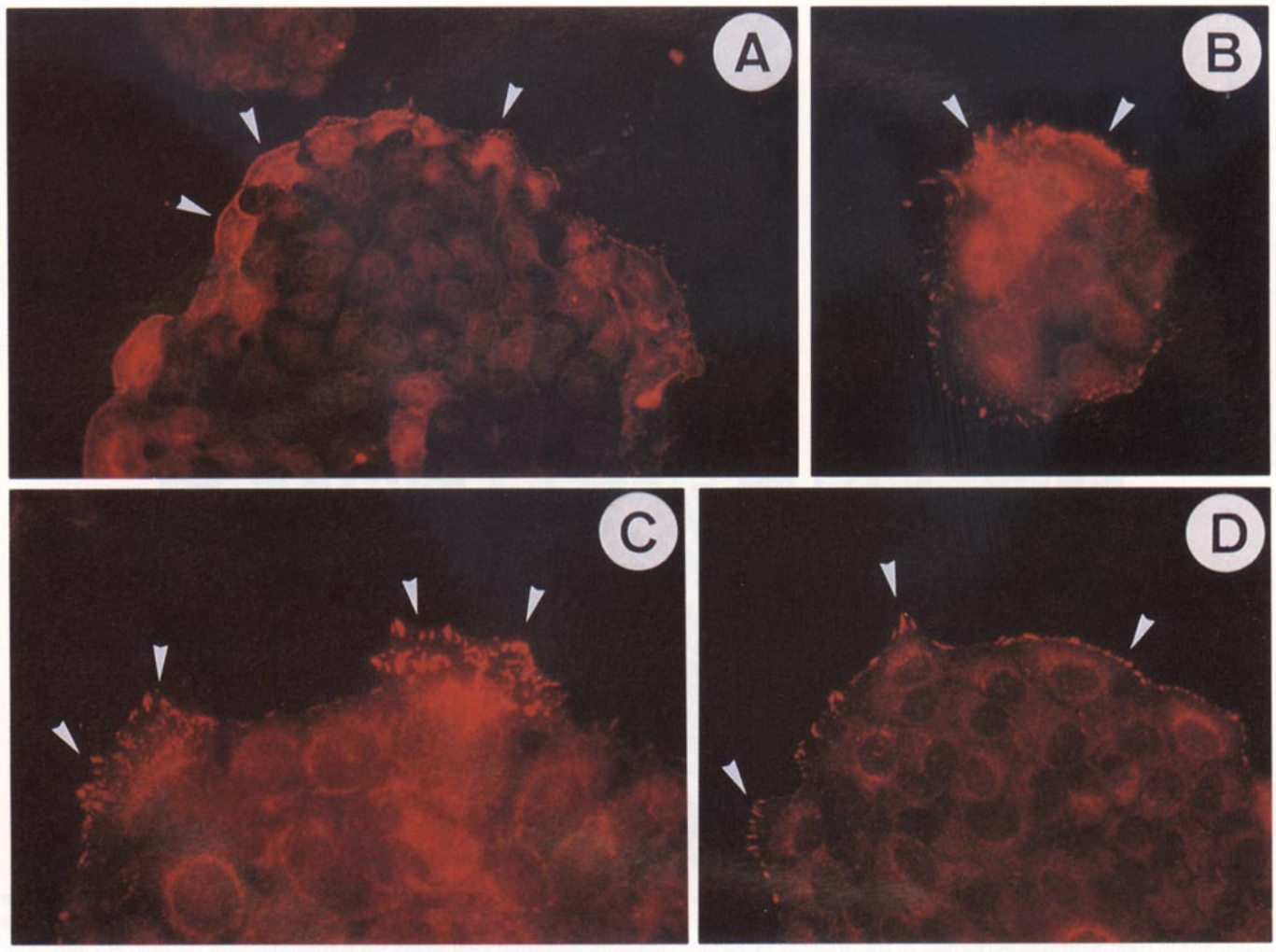

FIG. 9. Immunocytology revealed expression of uPA and uPA-R at the leading edge of outgrowing HaCaT cultures. HaCaT cells were cultured on glass coverslips for 24 h. Afterward, cultures were stained with goat anti-uPA IgG (A, B, C, arrowheads) or rabbit anti-uPA-R IgG (D, arrowheads). Microphotographs were taken using a Kodak Ektachrom 400× color slide film (magnification, ×592). No specific staining was observed when the anti-uPA or anti-uPA-R antibodies were omitted or when nonimmune IgG of the same species and concentrations were used (data not shown).

[52], which demonstrated the association of the uPA-R with cell/matrix contacts.

Furthermore, we provide evidence that the uPA-R of cultured keratinocytes is saturated with endogenous (pro-)uPA (Fig. 2D). This implies that during reepithelialization, where sheets of keratinocytes start to migrate out on a fibrin-rich extracellular matrix [45], the migrating keratinocytes do not depend on saturation of their uPA-R with (exogenous) (pro-)uPA provided via the interstitial fluid or from surrounding uPA-secreting cells [53]. This is also in agreement with the results of previous immunohistological studies of Grondahl-Hansen and co-workers [5], who demonstrated uPA-specific immunoreactivity in keratinocytes of the outgrowing epidermal sheet in wound healing.

PAI-1 and PAI-2 are the natural inhibitors of (u)PA [38, 39]. When HaCaT cells were exposed to PAI-1 or PAI-2, cell-associated plasminogen activation was inhibited in a dose-dependent manner (Fig. 6). The findings indicate that uPA-R-bound uPA is subject to inhibition by the specific PA inhibitors, which is in agreement with previous findings in myelo-/monocytes [29, 54], and epidermal cells [55]. For the human keratinocyte cell line NCTC Del Rosso and co-workers showed that the binding of PAI-1 to receptor-bound uPA enhances the internalization of the complex among enzyme, inhibitor, and receptor [55]. This may provide a pathway for down-regulation and clearing of cell-associated uPA. Whether in HaCaT cells the interaction of receptor-bound uPA with PAI-1 is followed by increased internalization remains to be explored.

Keratinocytes can produce both types of PAI *in vitro*

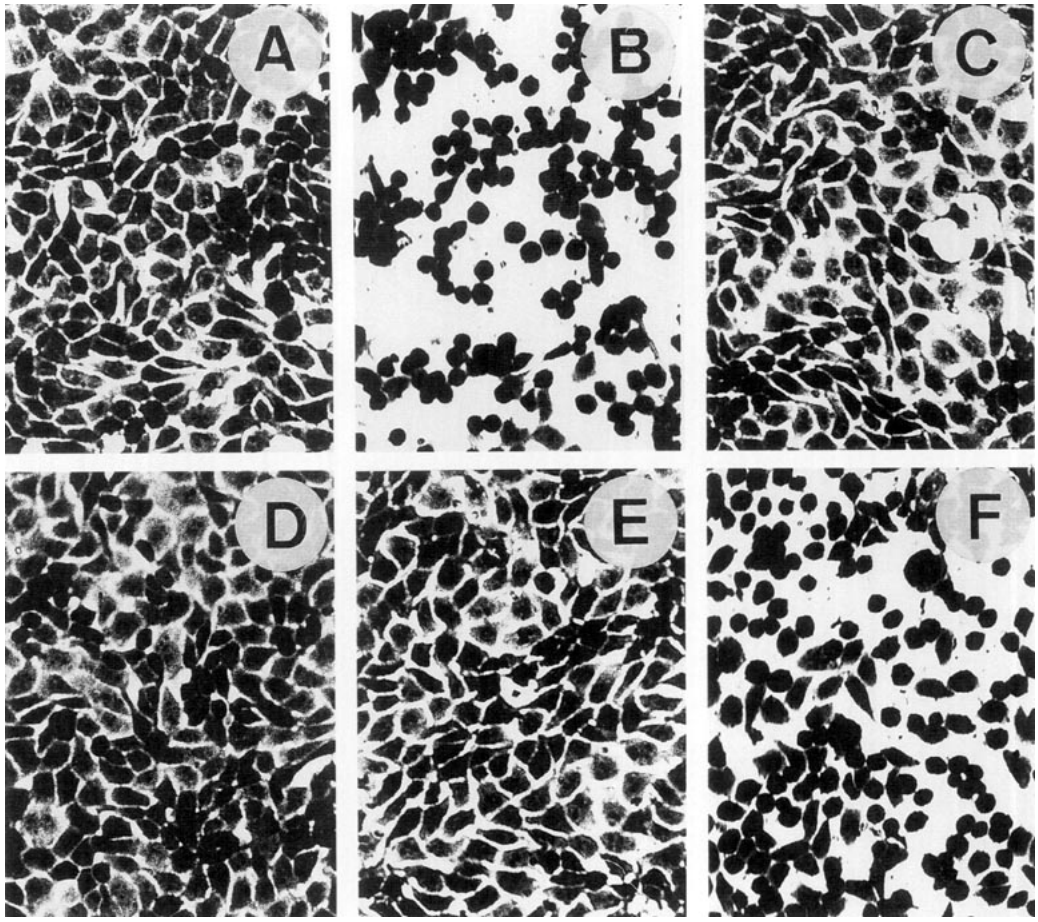

FIG. 10. Plasmin generated by cell-associated uPA interferes with HaCaT cell adhesion. HaCaT cells were grown to near confluence in regular 24-well tissue culture dishes. The cells were then incubated without (A) or with plasminogen (0.5 U/ml PBS; B–F) for 4 h at 37°C. The plasminogen treatment was performed in the presence of various additions, aprotinin (100 U/ml; C), tranexamic acid (15 m*M;* D), anti-catalytic uPA-specific antibody (10 μg/ml; E) or anti-catalytic tPA-specific antibody (10 μg/ml; F). The adherent cells were stained with methylene blue and microphotographs were taken. Addition and subsequent activation ensued by rounding and detachment of the cells from the culture plates; detachment could be prevented by the aprotinin, tranexamic acid, and an anti-catalytic uPA-specific antibody. Microphotographs were taken using a Kodak T-Max black and white film of 400 ASA (magnification: ×100).

[14]; *in vivo* the production of PAI-2 is characteristic of differentiated keratinocytes [40, 41]. Thus, it appears plausible that the production and secretion of PAI(-2) by differentiating keratinocytes could serve to down-regulate uPA bound to the uPA-R. The following scenario may be envisaged: in less differentiated keratinocytes, e.g., in keratinocytes of the primary epidermal outgrowth, uPA-R expression is up-regulated, followed by receptor saturation with endogenous (pro-)uPA. Concomitant binding of exogenous plasminogen to the cell surface would allow the start of plasminogen activation at the cell surface. Once epidermal continuity is restored and the keratinocytes begin to differentiate and synthesize PAI-2, the secretion of the inhibitor could then serve to down-regulate cell-associated uPA activity. This would favor the binding of keratinocytes to the underlying matrix and would be one aspect of the conversion of the keratinocyte into a more sessile phenotype. To get more insight in this matter, we are presently exploring the role of keratinocyte-derived PAIs in the regulation of surface-associated plasminogen activation.

Finally, there is increasing evidence that ligand binding to the uPA-R may have even wider implications. For keratinocytes the consequences of uPA-R/uPA interaction include the stimulation of proliferation [56] and the delivery of chemotactic signals [33]. For myelo-/monocytes an induction of differentiation has recently been demonstrated [57, 58]: in phorbol ester-treated myelo-/monocytes the autocrine interaction of uPA with the uPA-R mediated the conversion of the uPA-R-expressing cells into an adhesive phenotype. Whether the uPA/uPA-R interaction can also induce differentiation in keratinocytes remains to be explored.

The consequences of the uPA/uPA-R in keratinocytes may be dual: (i) the induction of the surface-associated pathway of plasminogen activation, as shown in the present report, and (ii) the induction of intracellular signals that may regulate the cell-biological behavior of the uPA-R-expressing keratinocyte [59, 60]. The former provides the cell surface and the pericellular space with the proteolytic potential of plasmin, permitting keratinocyte migration. The latter may regulate the subsequent behavior of the moving keratinocyte. The distinct consequences may have a phasic time course: an immediate, plasmin-dependent mobilization of the keratinocyte, followed by the chemotactically guided migration of the cells, and finally by the delivery of a signal for differentiation. These speculations provide further incentive for studies on the cell-biological consequences of uPA-R expression and ligand binding in human keratinocytes.

The study has been financially supported by the Deutsche Forschungsgemeinschaft (Kr 931/3-1). Part of the data has been presented in the doctoral thesis of J.R. that has been accepted at the Technical High School, Darmstadt, FRG. J.R. is indebted to Dr. H. G. Miltenburger (Department of Cell Biology, Technical High School, Darmstadt) for his support during preparation and processing of the doctoral thesis. The HaCaT cell line was kindly provided by Drs. Boukamp and Fusenig of the German Cancer Research Center. M.D.K. and J.R. are indebted to Dr. Klaus Rother (Heidelberg) for continuous and generous support.

REFERENCES

1. Danö, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. (1985) *Adv. Cancer Res.* **44,** 139–266.
2. Scully, M. F. (1991) *Br. J. Haematol.* **79,** 537–543.
3. Vassalli, J.-D., Sappino, A.-P., and Belin, D. (1991) *J. Clin. Invest.* **88,** 1067–1072.
4. Green, H. (1977) *Cell* **11,** 405–416.
5. Grondahl-Hansen, J., Lund, L. R., Ralfkiaer, E., Ottevanger, V., and Danö, K. (1988) *J. Invest. Dermatol.* **90,** 790–795.
6. Jensen, P. J., Baird, J., Morioka, S., Lessin, S., and Lazarus, G. S. (1988) *J. Invest. Dermatol.* **90,** 777–782.
7. Kramer, M. D., and Reinartz, J. (1993) *J. Clin. Invest.* **92,** 978–983.
8. Reinartz, J., Näher, H., May, H., and Kramer, M. D. (1993) *Arch. Dermatol. Res.* **284,** 432–439.
9. Jensen, P. J., Baird, J., Belin, D., Vassalli, J.-D., Busso, N., Gubler, P., and Lazarus, G. S. (1990) *J. Invest. Dermatol.* **95,** 13S–14S.
10. Gissler, H. M., Frank, R., and Kramer, M. D. (1993) *Br. J. Dermatol.* **128,** 612–618.
11. Reinartz, J., Batrla, R., Boukamp, P., Fusenig, N., and Kramer, M. D. (1993) *Exp. Cell Res.* **208,** 197–208.
12. Boukamp, P., Petrusevska, R. T., Breitkreuz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) *J. Cell. Biol.* **106,** 761–771.
13. Morioka, S., Lazarus, G. S., and Jensen, P. J. (1987) *J. Invest. Dermatol.* **89,** 474–477.
14. Hashimoto, K., Wun, T.-C., Baird, J., Lazarus, G. S., and Jensen, P. (1989) *J. Invest. Dermatol.* **92,** 310–315.
15. McNeill, H., and Jensen, P. J. (1990) *Cell Regul.* **1,** 843–852.
16. Jensen, P. J., and Wheelock, M. J. (1992) *Exp. Cell. Res.* **202,** 190–198.
17. Kramer, M. D., Vettel, U., Schmitt, M., Reinartz, J., Brunner, G., and Meissauer, A. (1992) *Fibrinolysis* **6,** 103–111.
18. Min, H. Y., Semnani, R., Mizukami, I. F., Watt, K., Todd, R. F., III, and Liu, D. Y. (1992) *J. Immunol.* **148,** 3636–3642.
19. Wohl, R. C., Summaria, L., and Robbins, K. C. (1980) *J. Biol. Chem.* **255,** 2005–2013.
20. Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961) *Arch. Biochem. Biophys.* **95,** 271–278.
21. Kramer, M. D., Schaible, U. E., Wallich, R., Moter, S. E., Petzoldt, D., and Simon, M. M. (1990) *Immunobiol.* **181,** 357–366.
22. Chomczynski, P., and Sacchi, N. (1986) *Anal. Biochem.* **162,** 156–159.
23. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Danö, K., Appella, E., and Blasi, F. (1990) *EMBO J.* **9,** 467–474.
24. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51,** 666.
25. Behrendt, N., Ronne, E., Ploug, M., Petri, T., Lober, D., Nielsen, L. S., Schleuning, W.-D., Blasi, F., Appella, E., and Danö, K. (1990) *J. Biol. Chem.* **265,** 6453–6460.
26. Barnathan, E. S., Kuo, A., Kariko, K., Rosenfeld, L., Murray, S. C., Behrendt, N., Ronne, E., Weiner, D., Henkin, J., and Cines, D. B. (1990) *Blood* **76,** 1795–1806.
27. Nielsen, L. S., Kellerman, G. M., Behrendt, M., Picone, R., Danö, K., and Blasi, F. (1988) *J. Biol. Chem.* **263,** 2358–2363.
28. Vassalli, J.-D., Baccino, D., and Belin, D. (1985) *J. Cell. Biol.* **100,** 86–92.
29. Cubellis, M. V., Andreasen, P. A., Ragno, P., Mayer, M., Danö, K., and Blasi, F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4828–4832.
30. Yodoi, J., Teshigawara, K., Nikaido, T., Fukui, K., Noma, T., Honjo, T., Takigawa, M., Sasaki, M., Minato, N., Tsudo, M. *et al.* (1985) *J. Immunol.* **134,** 1623–1630.
31. Mizukami, I. F., Vinjamuri, S. D., Trochelman, R. D., and Todd, R. F., III. (1990) *J. Immunol.* **144,** 1841–1848.
32. Ploug, M., Rönne, E., Behrendt, N., Jensen, A. L., Blasi, F., and Danö, K. (1991) *J. Biol. Chem.* **266,** 1926–1933.
33. Del Rosso, M., Fibbi, G., Dini, G., Grappone, C., Pucci, M., Caldini, R., Magnelli, L., Fimiani, M., Lotti, T., and Panconesi, E. (1990) *J. Invest. Dermatol.* **94,** 310–316.
34. Plow, E. F., Freaney, D. E., Plescia, J., and Miles, L. A. (1986) *J. Cell. Biol.* **103,** 2411–2420.
35. Mizukami, I. F., Garni-Wagner, B. A., DeAngelo, L. M., Liebert, M., Flint, A., Lawrence, D. A., Cohen, R. L., and Todd, R. F., in press.
36. Nykjaer, A., Petersen, C. M., Christensen, E. I., Davidsen, O., and Gliemann, J. (1990) *Biochim. Biophys. Acta* **1052,** 399–407.
37. Ellis, V., Behrendt, N., and Danö, K. (1991) *J. Biol. Chem.* **266,** 12752–12758.
38. Sprengers, E. D., and Kluft, C. (1987) *Blood* **69,** 381–387.
39. Andreasen, P. A., Georg, B., Lund, L. R., Riccio, A., and Stacey, S. N. (1990) *Mol. Cell. Endocrinol.* **68,** 1–19.
40. Hibino, T., Izaki, S., Ohkuma, M., Kon, S., Thorsen, S., and Astedt, B. (1988) *FEBS Lett.* **231,** 202–206.
41. Hibino, T., Izaki, S., and Izaki, M. (1986) *FEBS Lett.* **208,** 273–277.
42. Morioka, S., Lazarus, G. S., Baird, J., and Jensen, P. J. (1987) *J. Invest. Dermatol.* **88,** 418–423.
43. Stoppelli, M. P., Tacchetti, C., Cubellis, M. V., Corti, A., Hearing, V. J., Cassani, G., Appella, E., and Blasi, F. (1986) *Cell* **45,** 675–684.

44. Cubellis, M. V., Nolli, M. L., Cassani, G., and Blasi, F. (1986) *J. Biol. Chem.* **261,** 15819–15822.

45. Donaldson, D. J., and Mahan, J. T. (1988) *J. Invest. Dermatol.* **90,** 623–628.

46. Barnhart, M. I. (1968) *Biochem. Pharmacol.* **17,** 205–219.

47. Seppa, H. E. J., Yamada, K. M., Seppa, S. T., Silver, M. H., Klienman, H. K., and Schiffman, E. (1981) *Cell. Biol. Int. Rep.* **5,** 813–819.

48. Thiery, J. P. (1994) *Cell. Differ.* **15,** 1–15.

49. Miles, L. A., Dahlberg, C. M., Levin, E. G., and Plow, E. F. (1989) *Biochemistry* **280,** 9337–9343.

50. Pollanen, J., Saksela, O., Salonen, E.-M., Andreasen, P., Nielsen, L., Danö, K., and Vaheri, A. (1987) *J. Cell Biol.* **104,** 1085–1096.

51. Pollanen, J., Hedman, K., Nielsen, L. S., Danö, K., and Vaheri, A. (1988) *J. Cell Biol.* **106,** 87–95.

52. Del Rosso, M., Pedersen, N., Fibbi, G., Pucci, M., Dini, G., Anichini, E., and Blasi, F. (1992) *Exp. Cell Res.* **203,** 427–434.

53. Pyke, C., Kristensen, P., Ralfkiaer, E., Grondahl-Hansen, J., Eriksen, J., Blasi, F., and Danö, K. (1991) *Am. J. Pathol.* **138,** 1059–1067.

54. Ellis, V., Wun, T.-C., Behrendt, N., Ronne, E., and Danö, K. (1990) *J. Biol. Chem.* **265,** 9904–9908.

55. Del Rosso, M., Fibbi, G., Pucci, M., Dini, G., Grappone, C., and Nolli, M. L. (1991) *Exp. Cell Res.* **193,** 346–355.

56. Kirchheimer, J. C., Christ, G., and Binder, B. R. (1989) *Eur. J. Biochem.* **181,** 103–107.

57. Nusrat, A. R., and Chapman, H. A., Jr. (1991) *J. Clin. Invest.* **87,** 1091–1097.

58. Waltz, D. A., Sailor, L. Z., and Chapman, H. A. (1993) *J. Clin. Invest.* **91,** 1541–1552.

59. He, C.-J., Rebibou, J. M., Peraldi, M. N., Meulders, Q., and Rondeau, E. (1991) *Biochem. Biophys. Res. Commun.* **176,** 1408–1416.

60. Del Rosso, M., Anichini, E., Pedersen, N., Blasi, F., Fibbi, G., Pucci, M., and Ruggiero, M. (1993) *Biochem. Biophys. Res. Commun.* **190,** 347–352.

Received January 26, 1994
Revised version received May 20, 1994